GENETICS

Improving the Fixation Method for Preimplantation Genetic Diagnosis by Fluorescent In Situ Hybridization

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Purpose: Our purpose was to modify a fixation method using Tween-20 and HCl (TH) and to compare it with a protocol using methanol and acetic acid (MA) for the improvement of preimplantation genetic diagnosis by fluorescence in situ hybridization (FISH).

Methods: Single blastomeres were allocated to either the TH or the MA procedure. The two methods were compared to evaluate efficiency of fixation and the intensities of FISH signals.

Results: With the TH method, 123 (93.9%) of 131 blastomeres were fixed, while only 95 (78.5%) of 121 were fixed with MA. Average scores for the intensity of FISH signals were significantly stronger for TH than for MA ($P < 0.05$). There was also a significant difference in signal intensity scores between the two methods for type-3 nuclei.

Conclusions: Our results indicate that not only are fewer nuclei lost during fixation but also stronger FISH signals can be obtained with the TH method. Thus, modified TH can improve the overall efficiency of preimplantation genetic diagnosis.

KEY WORDS: fixation; fluorescence in situ hybridization; human embryos; preimplantation genetic diagnosis.

INTRODUCTION

Preimplantation genetic diagnosis (PGD) by fluorescent in situ hybridization (FISH) is a new and powerful

technique for detecting chromosomal abnormalities in human preembryos, with the goal of preventing severe genetic disorders (1,2). FISH techniques have been used for a variety of PGD purposes, including embryo sexing (3–5), aneuploidy screening for women of advanced maternal age (6–8), specific chromosome analysis for recurrent miscarriages, and detection of chromosomal structural abnormalities (9). However, the clinical application of PGD is still considered experimental because the FISH procedure is technically demanding, and the results can be inconsistent.

Because the decision to transfer an embryo is typically based on the diagnosis of a single embryonic cell, errors at any step of the FISH process can have important clinical consequences. Therefore, optimization of the FISH technique should result in significant improvements in PGD. Several factors can affect the integrity of FISH-PGD results, including the size and quality of chromosome-specific probes, the quality of the embryo/nucleus, and the fixation efficiency (10). An ideal fixation technique should be easy to learn and to perform, spread the nucleus well while maintaining nuclear (chromosomal) integrity, and minimize cytoplasm coverage so that DNA probes can penetrate nuclei easily during hybridization. Methods currently in use are based on an air-dry protocol developed more than three decades ago by Tarkowsky (11) and recently modified by Munne *et al.* (12). More recently, Coonen *et al.* (13) and Harper *et al.* (14) developed a new fixation method that used Tween-20 and HCl to lyse and spread the cells. However, no direct comparison of the methods has been reported, and we felt that the fixation methods could still be improved. Therefore, we modified the procedure developed by Coonen *et*

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al. (13) and Harper *et al.* (14) and compared it with the air-dry method to determine which of these methods produced optimal fixation efficacy and FISH signals.

MATERIALS AND METHODS

Arrested cleavage-stage human embryos were obtained on the third day of development from consenting in vitro fertilization patients. The zona pellucida was removed by exposing embryos in an acidified Tyrode's solution (pH 2.35) and rinsed three or four times in $1 \times$ phosphate-buffered saline (PBS) supplemented with 1 mg/ml bovine serum albumin (BSA) (Sigma, St. Louis, MO). If the embryos showed signs of compaction, they were placed in a Ca^{2+} - and Mg^{2+} -free Hank's medium (GIBCO, NY) supplemented with 1.0 mg BSA/ml for 5–10 min at room temperature. Single blastomeres were then isolated by pipetting the zona-free embryos through a small-pore pipette (80–100 μm inner diameter) with the tip polished to facilitate the breakage of cellular connections.

Individual blastomeres from one embryo were randomly divided and allocated to one of two fixation procedures: the air-dry "MA" (Methanol:Acetic acid) method (11,12) and the modified "TH" (Tween-20, HCl) method (13,14). No more than four cells from the same embryo were fixed onto a slide. A single investigator performed all fixations.

The air-dry (MA) method used methanol and acetic acid (3:1) as originally described (11) and as modified for single blastomere fixation (12). Individual blastomeres were briefly exposed to a hypotonic solution (0.068 M KCl, supplemented with 1 mg/ml BSA (Sigma) for 1–2 min, then transferred onto a clean slide. Freshly prepared methanol:acetic acid (3:1) was dropped over the cell while the hypotonic solution evaporated, as observed under a dissecting microscope. Additional fixative could be added if cell lysis was not apparent.

The original procedure (13,14) was modified and performed as follows. After single cells were obtained, they were briefly exposed to a hypotonic solution (described above) to permit some cell expansion. The concentration of HCl used in the spread solution was reduced from originally 0.01 to 0.001 N (J. T. Baker, NJ), whereas the concentration (0.1%) of Tween-20 (Sigma, MO) was unchanged. Individual cells were then placed onto a clean slide with a small amount ($<1 \mu\text{l}$) of hypotonic solution. Spreading solution was expelled around the blastomere with a small-pore pipet (approximately 60 μm in inner diameter), while the

process was continuously monitored under a dissection microscope until cell lysis and nucleus exposure. After identifying the locations of the nuclei, the slides were air-dried, then washed in $1 \times$ PBS for 5 min in a Coplin jar at room temperature. They were further dehydrated in serial 70, 85, and 100% ethanol. Pepsin treatment, which was used in the original process to digest cytoplasm, was not needed for more than 95% of cells in our study because only a few nuclei were covered with insignificant amounts of cytoplasm.

Images of the fixed nuclei were captured by a Quips LS imaging system (Vysis, IL), and the quality of nucleus was classified according to the following morphological characteristics: type 1 included fragmented or broken nuclei, or those with a significant part of nuclear material scattered close by, type 2 included those with expanded or enlarged nuclei with intact boundaries, and type 3 included those with clearly intact and compact or slightly expanded nuclei. Figure 1 illustrates the typical morphology of these three types.

To evaluate the efficiency and efficacy of the two methods, two parameters were compared. The percentage of cells successfully fixed or lost was recorded, and the average FISH signal intensity was obtained using two sets of probes. In the first set, directly labeled chromosome-13 and -21 LSI probes (Vysis) were used. In the second set, directly labeled chromosome-X, -Y, -18 CEP probes (Vysis) and a digoxigenin-labeled chromosome-16 probe (Oncor, MD) were used. FISH procedures were the same as described in the manufacturer's instructions (Vysis). FISH images were captured by a Quips LS imaging system (Vysis). A FISH signal score was assigned to each captured image, according to the following criteria: 5, signals were very strong and bright; 4, signals were strong and clear allowing a clear analysis; 3, signals were clear and diagnosis could be made without further color adjustment; 2, signals were weak on the original captured images, but diagnosis could be ascertained by a color adjustment; 1, signals were so weak a diagnosis could be made only by a color adjustment; and 0, no signal, even after color adjustment. For each technique, an average score by nuclear morphology type was calculated. Examples of FISH images are shown in Fig. 2.

The Mann-Whitney *U* test was used to detect differences, and statistical significance was defined as *P* less than or equal to 0.05. The study was approved by the Institutional Review Board of Cornell University Medical College.

Fig. 1. Examples of three nuclear types. (a) Typical morphology of the type-1 nucleus, showing a disruptive and fragmented nucleus. (b) Typical morphology of the type-2 nucleus, showing an expanded nucleus with slightly disruptive boundary. (c) Typical morphology of the type-3 nucleus, showing an intact nuclear structure with clear boundary.

RESULTS

From a total of 41 arrested embryos, 252 blastomeres were isolated and prepared for fixation. Of those, 123 (93.9%) of 131 cells were fixed successfully by the modified TH method, and 95 (78.5%) of 121 cells were fixed successfully by the MA method. Significantly more cells were lost during fixation by the MA method than by the modified TH method (21.5 vs. 6.1%; $P < 0.05$).

Morphologically, there were significant differences in the distribution of nuclear types among the three

nuclear groups as shown in Fig. 3. More than half of the nuclei (52.6%) were classified as type 3 when the modified TH method was used for fixation, compared with one-fifth (19.4%) when the MA procedure was used. In addition, 48.3% of the nuclei were classified as type 2 in the MA fixation group and 18.4% in the modified TH group. No significant difference was detected between the two groups in the proportion of type-1 nuclei (28.9% for modified TH vs. 31.7% for MA).

As anticipated, FISH signal scores were lower with LSI probes because of the small size of the probes

Fig. 2. Examples of FISH images. (a) An example of FISH image with LSI 13 chromosome-13- and -21-specific probes (four orange dots representing 2×13 , and 2×21). (b) An example of FISH image of a nucleus with XX, 2×16 , 2×18 (X = yellow, 16 = red, and 18 = green). (c) An example of FISH image of a nucleus with XY, 2×16 , 2×18 [X = yellow, Y = aqua (12 o'clock), 16 = red, and 18 = green].

Fig. 3. The distribution of nuclear types by the two fixation methods. Numbers in parentheses represent the numbers of nuclei fixed.

compared with those using CEP and digoxigenin-labeled satellite probes. In both groups, FISH signal intensity tended to be stronger when the modified TH method was used for cell fixation, although the difference was not significant. There was a significant difference, however, in the signal intensity between the two fixation groups when the two probe-type groups were combined (Table I).

The relationship between the nuclear morphology and the average FISH signal intensity scores for the two fixation methods are summarized in Table II. In all three types of nuclei, signal intensity scores were higher for modified TH than for MA, although a significant difference was detected in type-3 nuclei only.

DISCUSSION

Because it relies on the genetic analysis of single cells, the FISH-PGD process is quite sensitive to errors at any step in the procedure. Therefore, even a small improvement in this process can be clinically significant, resulting in an increase in the number of embryos diagnosed correctly and subsequently made available for successful transfer. The central purpose of this study was to compare two FISH-PGD fixation meth-

Table II. Signal Intensity Scores (Mean $\pm$ Standard Error of the Mean) by Two Fixation Methods and Nuclear Types

Fixation method	Number of subjects	Nuclear morphology		
		Type-1	Type-2	Type-3
TH ^a	76	2.23 $\pm$ 0.30	2.79 $\pm$ 0.33	3.38 $\pm$ 0.19 ^b
MA	60	2.16 $\pm$ 0.40	2.41 $\pm$ 0.26	2.67 $\pm$ 0.26 ^b

^a Modified.

^b Between-fixation group difference is statistically significant ($P < 0.05$).

ods: the conventional air-dry process, MA (11,12), and a modified version of the recently developed fixation protocol, TH (13, 14).

We made three modifications to the TH protocol. First, we introduced a hypotonic treatment similar to that used for the MA method. The brief exposure to the hypotonic solution promoted subsequent membrane disruption. Second, the acid solution was reduced from 0.01 to 0.001 *N* to attenuate cell damage. Finally, because the cells had been treated with hypotonic solution prior to cell spreading, very few nuclei (<5%) were covered by cytoplasm; therefore, in a majority of cells (>95%) an enzymatic treatment with pepsin was unnecessary. In fact, we observed, in a preliminary study, that pepsin treatment damaged nuclear structure when the concentration (100 μ g/ml) (13,14) was used. Furthermore, when we reduced the pepsin concentration from 100 to 10 μ g/ml; a weaker FISH signal intensity was observed comparing those without pepsin treatment (data not shown). Finally, obviating this step should reduce the time needed for the completion of the fixation process.

In this study, more cells were successfully fixed with the modified TH method (94%) than with the standard MA method (79%). It should be noted that throughout the study, a single investigator, who was initially unfamiliar with both the MA and the modified TH fixation procedures, completed all fixations. Consequently, the differences in success rates between the two fixation methods may be accounted for by the relative ease with which the modified TH method was learned and performed, an important characteristic that can enhance FISH-PGD efficiency. It should also be noted that the MA method appears less tolerant to variations in procedure, although 90 to 95% of the cells can be fixed successfully with experienced hands. For example, the time window for the addition of the MA fixative is extremely narrow, increasing the chances for error. If the MA fixative is added too early, i.e., before the hypotonic solution dries out, the risk

Table I. Average Signal Intensity Scores Obtained by the TH^a and MA Fixation Methods

Fixation method	Number of subjects	FISH probe		Average score ^a
		13 and 21	XY, 16, 18	
TH ^b	76	2.38 (45) ^c	3.74 (31)	2.93 $\pm$ 0.15 ^d
MA	60	1.97 (32)	2.82 (28)	2.37 $\pm$ 0.18 ^d

^a Modified.

^b Mean $\pm$ standard error of the mean.

^c Numbers in parentheses represent the number of cells fixed in each group.

^d Between-fixation group difference is statistically significant ($P < 0.05$).

of losing the nucleus will be high. Conversely, if the addition of the fixative is delayed and the cell becomes too dry, fixation will be less than optimal because the cytoplasm may cover the nucleus.

Although the criteria used for nuclear grading may not be a true reflection of quality, there was an apparent linear relationship between the nuclear grade and the FISH signal scores in both fixation groups. Among the three types of nuclei, the strongest signals were seen in type 3. When the two fixation methods were compared, a better nuclear morphology was obtained for type-3 nuclei by the modified TH method, which, in turn, produced better FISH signals using either LSI or CEP probes. The reasons for the better signal scores with the TH method may be related to the extent of cytoplasm coverage. We noticed that in the TH group, cytoplasm appeared to be pushed aside or diminished when the spreading solution was added. With MA-fixed nuclei, in contrast, some cytoplasm covered the nuclei, either preventing probe penetration or reducing the reflected fluorescent signals during microscopic analysis.

In summary, optimization of the FISH procedure can improve the overall efficiency of PGD. Our results demonstrate that the modified TH method appears easier to learn and to perform, and displays a higher fixation success rate. More important, stronger FISH signals can be obtained using this method. Taken together, these results suggest that the modified TH method of fixation may improve the PGD process with FISH.

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